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Abstract

The determination of functional groups on the microgram
scale (ca. 50μg samples) which was initiated as described
in the previous annual report, has been continued.

The present report describes methods which have been
developed for the determination of :

    I   Sulfhydryl groups.
   .II Nitroso and other nitrogen-containing groups.

   III Vicinal glycols (Malaprade reaction).
   IV Olefinic unsaturation.

Sulfhydryl Groups.

Macro or micro determinations of mercapto groups are
generally based on iodimetric or argentometric titrations.
Argentometric methods usually require an amperometric or
potentiometric method of end-point detection - a technique
which is awkward in manipulation on the submicro scale.
Accordingly, attention was given first to the iodimetric
methods. The actual titration of iodine with thiosulfate
to starch indicator was very accurate on the scale of working
and up to 30% of ethanol could be present in the solution
without affecting the end-point significantly - an important
point in view of the difficulty of dissolving many mercaptans
in water. The proposed method was to react the mercaptan
with excess iodine, which would then be back-titrated with
thiosulfate. It was shown that reduced glutathione could be
determined accurately (± 2% relative) with a reaction time of
1 minute. However, other compounds tested gave very inaccurate
and not readily explicable results, and it was thought un-
profitable to pursue the iodimetric studies farther.

Attention was then turned to mercurimetric methods,
of which the best seemed to be that of Fritz and Palmer
(Anal.Chem., 1961, 33, 98). In this method, the mercaptan

in acetonic-pyridine medium is titrated with mercuric
perchlorate solution to thio-Michler's ketone indicator.
It was shown that the method could be scaled down to deal
with 50μg samples without too much trouble. However, an
80% ethanolic medium tended to give more reliable end-points
than an acetonic medium on the submicro scale, particularly
in the case of blank determinations. A wide variety of
samples were analysed and typical results are given in
Table I.

TABLE I

Compound	Weight taken in ug.	Volume of mercuric perchlorate consumed in ul.	% SH		% Error	% Recovery
			found	expected		
L-cysteine-HCl	52.401	32.0	20.77	20.94	-0.17	99.19
	55.702	34.0	20.76		-0.18	99.14
	83.329	50.6	20.64		-0.30	98.57
Thionalide	92.684	40.0	14.68	14.54	+0.14	100.96
	72.863	31.0	14.47		+0.07	99.52
o-mercapto-benzoic acid	55.355	35.0	21.50	21.45	+0.05	100.23
	68.953	42.4	20.91		-0.54	97.48
2-mercapto-imidazole	76.260	73.0	32.55	33.02	-0.47	98.58
	81.235	80.0	33.49		+0.47	101.42
2-mercapto-benzimida-zole.	64.564	41.0	21.59	22.02	-0.43	99.05
	72.127	46.0	21.69		-0.33	98.50
	75.071	49.0	22.20		+0.18	100.82
2-mercapto-benzothia-zole.	80.906	46.0	19.33	19.77	-0.44	97.77
	61.061	36.0	20.05		+0.28	101.42
	59.488	35.0	20.01		+0.24	101.21

In general the overall accuracy of the method was ±0.5% absolute.
For pure glutathione which was used as a standard compound,
the average recovery on 12 determinations was 99.3% ± 3%.
Several semi-solid and liquid samples were analysed with
excellent results; in such cases, a solution was prepared from
which aliquots were taken to contain ca 50μg of substance.
Moderate amounts of sodium chloride and bromide interfered
slightly, while iodide and cyanide interfered seriously.

Organically bound chlorine did not interfere, nor did
sulphides and disulphides. The interferences thus seem to
be about the same as on the larger scales of working used by
Fritz and Palmer.

The use of diphenylcarbazone as indicator instead of
Thio-Michler's ketone was also examined, since the former was
known to be a good mercury(II) indicator on the submicro
scale. A micro method has been described by Gregg et al.
(Anal.Chem., 1961, 33, 269), which appeared to be quite
straightforward. When the procedure was scaled down, the
end-point was found to be rather sharper than that obtained
with Thio-Michler's ketone, and excellent results were
obtained with a good range of compounds. However, in a few
cases, the stoichiometry of the reaction was different, the
complex formed in the titration having a structure containing
1 Hg : 1 SH, instead of 1 Hg : 2 SH as happened in all cases
when Thio-Michler's ketone was used as indicator, and in the
great majority of cases when diphenylcarbazone was used as
indicator. An attempt was made to use dithizone as indicator,
being intermed_ate in structure between diphenylcarbazone and
Thio-Michler's ketone, but this was completely unsuccessful
because dithizone itself was titrated quantitatively. The
explanation of the anomalous results obtained with diphenyl-
carbazone indicator for reduced glutathione, cysteine hydro-
chloride and 2-mercaptopyrimidine appears to be somewhat
esoteric and need not be detailed here.

The diphenylcarbazone procedure appears to be preferable
where it can be applied with certainty, but the Thio-Michler's
ketone procedure is the more reliable when the sample is
unknown.

EXPERIMENTAL

Thio-Michler's ketone procedure. The weighed sample (40
- 80 μg) was dissolved in 0.2 ml distilled water and 0.8 ml
ethanol, both air-free and introduced from hypodermic syringes;

the order of addition of the two solvents depends on the
solubility of the sulphhydryl compound. The test tube was
then clamped over the white tile on the titration platform
and the stirring bar was introduced. To the sample solution,
5 micro drops (ca. 0.08 ml) of pyridine buffer (pH6) and
3 micro drops (ca. 0.04 ml) of Thio-Michler's ketone indicator
(0.01% in acetone) were added. The solution was left stirring
for 30 seconds and the burette tip of the mercuric per-
chlorate solution was introduced. The bright yellow colour
was then titrated under the "daylight" lamp until it almost
vanished. Towards the end-point small increments of 0.2 µl
of the mercuric solution was added till the appearance of the
first blue colour facilitated by the white background.

Notes.

(i)   For mercaptans which are insoluble in the ethanol-
      water mixture, use a similar volume of acetone where
      the starting colour is yellowish vivid-green and goes
      to a blue colour with slight greenish tint at the
      end-point.

(ii) Acidic mercaptans must be neutralised in non-acetone
      media (e.g. in ethanol or water solutions) with
      0.02N sodium hydroxide added from an Agla syringe to
      just light pink colour of phenol-phthalein (one micro
      drop (ca. 0.01 ml) of 0.1% ethanol solution).

(iii) Blank determinations must be run in exactly the same
      way using the same amounts of reagents, but omitting
      the sample; the blank values found in the present work
      were of the order of 2 - 4 µl of 0.005 M. mercuric
      perchlorate solution.

Diphenylcarbazone procedure. The weighed sample (40 - 80µg)
was dissolved in 0.2 ml distilled water and 0.8 ml ethanol,
both air-free and introduced from hypodermic syringes;
either the water or the ethanol was added first depending on

the solubility of the mercaptan. To the sample solution, 1 micro drop (ca. 0.015 ml) of bromophenol blue (0.03% aqueous), 1 micro drop (ca. 0.02 ml) nitric acid (0.05N) and 3 micro drops (ca. 0.03 ml) diphenyl-carbazone (0.5% ethanolic) were added while the test tube was standing clamped on the white tile and the solution was being stirred. The burette tip of the mercuric perchlorate solution was then inserted and the solution was then titrated under the illumination of a "daylight" electric lamp till the first sign of a reddish tinge, i.e. till the original bright yellow colour was just not restored. Increments of 0.2 µl were used towards the end-point and white sheet of filter paper standing behind the test tube helped in detection of the excellent end-point.

Notes.

(i)   Ethanol alone can be used as solvent with which a sharp end-point was also available; while with acetone the end-point was not satisfactory.

(ii) There is no need for the neutralisation of acidic mercaptans as acetone is not involved at all in the above procedure.

(iii) Blank determinations were carried out following exactly the same procedure but without the sample. The usual blank value obtained was 1 - 2 µl of 0.005M mercuric perchlorate solution.

II. Nitroso and other nitrogen-containing linkages.

Nitroso groups : The method described in the last report for the determination of nitro groups based on reduction with titanous solution was shown to be directly applicable to the determination of nitroso groups on the microgram scale. This was expected.

Azoxy groups : Little information seems available in the
literature on determination or reduction of azoxy groups
with titanous solution. Only 2 pure samples were available
for the present work : azoxybenzene and p-azoxyanisole. The
p-azoxyanisole was analysed by the procedure for nitro-
groups and gave excellent results based on a consumption of
4 Ti^{3+} per mole of compound, i.e. a straightforward reduction.
However, results for azoxybenzene were extremely variable
depending on the time of reduction; reasonably good results
were obtained after a 20-min. reaction time, but obviously
the reduction takes complicated pathways and the procedure
can scarcely be recommended as generally applicable.

Azo groups. On the macro and micro scales, azo groups have
often been determined either by direct titration with
titanous solution or by reaction with titanous ion followed
by titration of its excess. In the present work, several
alkyl azoates were analysed by the general nitro procedure
on the microgram scale without difficulty, excellent results
being obtained on the basis of 2 equivalents of titanous
consumed, which argues a diphenyline or semidine re-arrangement.
Azo linkages are usually reduced in highly acidic media, but
in the present work it was found that azobenzene was reduced
much more rapidly in the citrate-buffered solution used in
the general procedure for nitrogroups. Excellent results
were obtained after a 5-min. reaction time, but results were
very high and did not appear to correspond to any
stoichiometric reaction after 10-min. reduction or longer.
After 5 min. reduction with benzidine re-arrangement had
occurred, 2 equivalents of titanous solution being required.
It was found that m-azotoluene and bis(trifluoromethyl)
azobenzene could not be reduced at all, even on prolonged
reaction in acidic or buffered media. Such behaviour can be

explained on the basis of the substituents hindering proton-
ation and thus electron transfer (cf. Hinshelwood et.al.,
J.Chem.Soc., 1953, 3384; 1954, 2736; 1956, 620).

Taken as a whole, the results showed that no general
technique for determination of azo groups can be based on
reduction with titanous solution, although most compounds
can be determined with reasonable accuracy if the structure
is known. Obviously, the method would have no value for
diagnostic purposes.

Hydrazo groups : Possible reduction of several compounds
with titanous solution under a variety of conditions was
examined. Under no circumstances did the reduction proceed
quantitatively in a stoichiometric reaction. Several
hydrazine compounds were also studied. It was found, in
confirmation of results on other scales of working, that the
hydrazine group alone was not reduced, but that when nitro
groups were also present, the nitro group was reduced to
the corresponding amine while the hydrazine linkage was reduced
to the amine and ammonia. Thus, very good results could
be obtained for compounds such as 2,4-dinitrophenylhydrazine
and cyclohexanone-2,4-dinitrophenylhydrazine.

Oxime groups: A few oximes were examined to see if they
could be reduced, since there is no report of such reduction
in the literature. With some compounds, such as pyridine-
2-aldoxime, reduction to the amine was quantitative but with
other compounds, there was no reduction.

Conclusion : These studies on the reduction of nitrogen-
containing linkages by titanous solution indicate that general
procedures are possible for the determination of nitro and
nitroso groups, excellent results being obtained even with
30-70 µg samples. For the other linkages examined (azo,
azoxy, hydrazine and oxime), certain compounds can be

determined accurately, but with unknown materials there can
be no certainty about the results.

Experimental detail for these determinations was given
in the previous Annual Report.

Periodate Oxidations (Malaprade Reaction)

Periodate oxidations are very useful in carbohydrate
analysis, giving a good deal of information on structure from
a very simple determination. Virtually all the work done on
this area has been on the macro-scale, apart from a few
biochemical studies. It was, however, considered valuable
to develop a suitable procedure for microgram samples.

In the macro methods, it is usual to react the carbo-
hydrate with excess of periodate, and to determine the excess
of periodate, as well as one or more of the reaction products,
usually a simple aldehyde or organic acid. With the simpler
carbohydrates at least, the products usually include formalde-
hyde or acetaldehyde and/or formic or acetic acid.

In the present work concerned with scaling the methods
down to the 50μg range, it was quickly shown that the reactions
proceed essentially as on larger scales of working. No
difficulty was found in titrating the excess of periodate
used, a direct titration with arsenite solution to a Thyodene
end-point proving most satisfactory. This method was preferred
to a thiosulphate titration, because periodate was reduced
only to iodate in the bicarbonate medium employed, thus
improving the blank : sample ratio.

It was found that for simple carbohydrates such as
mannitol and sorbitol, a reaction time of 15 min. is suffic-
ient; as on larger scales, more complex materials, and
particularly cyclic compounds, take a longer time for degrad-
ation. In general, the time of reaction and, where appropriate,
incomplete reaction to a defined product, were found to follow
closely the data available for macro analysis.

On the macro-scale it is normal practise to take aliquots
of the periodate-treated solution for determination of excess
periodate. Aliquotting presents some difficulties in submicro-
scale analysis and, accordingly, the methods were designed so
that as many as possible of the required steps could be
carried out consecutively on the same solution.

The common acid formed in the oxidations is formic acid.
It was found possible to titrate this acid before the titration
of excess periodate, when methyl red was used as indicator,
and suitable precautions were taken. An attempt to use an
iodimetric titration of the acid failed because of incomplete
reaction at the dilution involved, no matter which modification
was applied.

The aldehyde formed in the reaction is generally
determined by a spectrophotometric method involving chromo-
tropic on a separate aliquot, or by an iodimetric bisulphite
procedure after separation by distillation. Attempts to
apply distillation techniques were a complete failure on the
submicro scale. Accordingly, the hypoiodite oxidation method
of Romijn for formaldehyde was examined, for it was thought
that this method could be applied to the solution after the
titration of excess of periodate. It was shown that under
controlled conditions, the hypoiodite method could give
quite good results for microgram quantities of formaldehyde
(and even acetaldehyde when appropriate corrections were made).

It was finally shown that it was quite feasible to run
3 consecutive determinations of acid, excess periodate and
aldehyde on the same solution by the following procedure.

Procedure.

30 - 80 µg of the sample were weighed into the reaction
vessel, and 150 µl of periodate solution was added, the
tip of the burette being washed with 2 micro drops of water.
The tubes were then stoppered and stored in a cool dark place
until reaction was complete.

50 µl of 0.01% methyl red indicator were then added
either from a dropper or an Agla burette, followed by a
rotor and 100.0 µl of standard sodium hydroxide (0.025N).
The tip of the burette was raised above the liquid surface
and washed with 1 drop of water. The hydrochloric acid
burette was then introduced, the tip also dipping about 1 mm.
under the liquid surface. The excess of alkali was titrated
to the disappearance of the yellow colour of methyl red (red-
brown end-point).

The burette tip was raised above the liquid surface and
washed with 1 drop of water. Then approx. 0.1g of sodium
bicarbonate was added, followed by 1 drop of potassium iodide
solution, and the liberated iodine was titrated with standard
arsenite solution the "thyodene" indicator being added soon
after the start of the titration, and the end-point being
taken when the brown-purple colour completely disappeared
leaving a clear yellow solution.

Sufficient 4N hydrochloric acid to destroy the
bicarbonate and iodate was then added (preliminary tests
showed that 1 drop of the acid solution used was sufficient
for this purpose; this amount left only a very small excess).
The solution was then carefully rebuffered with sodium
bicarbonate (approx. 0.015g.), and the liberated iodine was
titrated with arsenite solution until the solution was
completely clear yellow. Quickly 6 drops of approx. 4N
sodium hydroxide and 50 µl of approx. 0.1N iodine were
added, in that order, the solution being vigorously stirred
during the addition. The resulting solution was then placed
in a cool dark place after the tube had been stoppered.

After exactly 30 minutes sufficient 4N hydrochloric
acid was added to liberate the iodine and make the solution
slightly acidic (ca. 6 drops). The liberated iodine was
titrsted with standard 0.025N sodium thiosulphate to the first
clear red end-point.

Blank titrations were carried out in exactly the same way.

About 10 relatively simple carbohydrates were tested using the above method. The results shown in the following table are fairly typical of the results obtained.

Dulcitol. M.W. 182.17.
 a) Formic Acid Determination.

	Wt.Sample μg.	Alkali Titre Theory μl.	Alkali Titre Expt[l]. μl.	% Recovery
NaOH 0.02537N	55.04	47.64	46.09	96.8
	72.94	63.13	61.74	97.8
	69.77	60.38	59.13	97.9
	44.36	38.39	38.09	99.2

b) Periodate Consumption.

	Wt.Sample μg.	Arsenite Titre μl.	Wt. Sample Expt[l]. μg.	% Recovery
Arsenite 0.05027N	55.04	58.3	53.39	97.0
	72.94	77.9	71.33	97.8
	69.77	75.7	69.32	99.4
	44.36	47.3	43.31	97.6

c) Formaldehyde determination.

	Wt.Sample μg.	Wt.HCHO Theory (μg.)	Titre of Thio μl.	Wt.HCHO Expt[l].μg.	% Recovery
Thio: 0.02576N	55.04	18.13	44.0	17.00	93.8
	72.94	24.03	60.0	23.18	96.5
	69.77	22.98	60.4	23.34	101.6
	44.36	14.61	34.0	13.14	90.0

A number of acid-sensitive materials required analysis, and it was shown that the submicro oxidation was also satisfactory in bicarbonate medium. Of course, no determination of acid formed was then possible, but the remainder of the procedure offered no difficulty.

As a general rule the maximum errors to be expected from the 3 steps are :

±2% relative for periodate consumption,

±2% relative for acid determination, and

±10% relative for formaldehyde determination.

These figures are of the same level as those generally
found on the macro scale. The error in the aldehyde
determination seems large, but the results are in fact quite
accurate enough for diagnostic purposes. It is interesting
to point out that these three consecutive analytical steps
would be very difficult to achieve on larger scales of
working.

An examination of more complex compounds, e.g.
∝-methyl-D-glucoside and 1,2,5,6-diisopropylidene-D-mannitol,
by the above procedure, showed that while the acid and excess
periodate determinations proceeded according to expectations,
the determination of formaldehyde did not. Several compounds
which produced no formaldehyde or acetaldehyde in the oxidation
with periodate still consumed alkaline hypoiodite, the large
aldehydic moeities formed being determined quite accurately.
Thus, although all the results could be explained on a
post mortem oasis, and such experiments could yield data
of a useful confirmatory nature, the hypoiodite method was
obviously of little value for analysis of complicated unknown
carbohydrates.

Accordingly, it was decided to examine the spectro-
photometric method for the determination of formaldehyde,
based on reaction with chromotropic acid - a reagent which is
very selective for formaldehyde among the various aldehydic
moeities which might be formed. A scale-down of the normal
macro technique proved quite satisfactory and could be applied
to the solution from reaction with periodate directly after
the titration of excess periodate. Generally, the results for
formaldehyde were better when the oxidation was done in a
bicarbonate medium. The following method was found satis-
factory.

Experimental

Reagent : Chromotropic acid solution. Dissolve 1.00 g. of
chromotropic acid in distilled water and filter. Add 300 ml
of concentrated sulfuric acid to 150 ml of distilled water,
cool and add to the chromotropic acid solution, diluting to
500 ml. Store in a brown glass bottle and re-prepare every
week.

Procedure: A suitable sample weighing between 30 and 80 µg
was placed carefully in a clean dry 5 ml graduated flask,
the sides of the flask being gently tapped to ensure that all
the sample reached the bottom. Approximately 0.01 g sodium
bicarbonate was added carefully by means of a moderately
heaped micro spatula. The sides of the flask were then
washed down with 0.5 ml of water, and the contents of the
flask gently swirled to dissolve the bicarbonate and sample.
Then 150µl, approximately 0.05N sodium periodate, was added,
the tip of the burette being washed with 0.1 ml water. The
flask was stoppered and placed in a cool dark place until
reaction was complete.

After this time 1 drop of 10% potassium iodide was added
with a magnetic stirrer, and the liberated iodine titrated with
standard sodium arsenite solution, the tip of the burette
dipping slightly below the liquid surface. When the iodine
colour had almost completely disappeared, 1 micro drop of starch
solution was added and the titration continued until the
solution was completely clear. The burette tip was then
raised above the liquid surface and washed with a few drops
of distilled water.

The magnetic stirrer was removed from the liquid with
a magnet outside the flask, being washed with about 1 ml
of water when halfway up the neck of the flask.

From a pipette 2.0 ml of 0.05N arsenite solution were

then added and the resulting solution shaken occasionally for about 10-15 minutes. The solution was then diluted to 5 ml.

1 ml of the resulting solution was pipetted into a 25 ml graduated flask, or any other ∼25 ml container of approximately the same dimensions.

10 ml of chromotropic reagent solution was then added, again from a pipette, and the resulting solution shaken gently to mix. The flask was then loosely stoppered and placed in a vigorously boiling water bath for 30 minutes. The water bath should preferably be in a dark corner where light or direct sunlight cannot fall on the reaction flasks. Blank determinations were carried out in exactly the same way.

Colour Measurement.

The colour was measured on a spectrophotometer at 570mμ using a 1 cm. cell. The optical density for a blank solution carried through the same procedure was also measured, although in most cases this was zero, i.e. 100% transmittance.

The weigh of formaldehyde was determined by reference to a previously prepared calibration curve using mannitol as the standard.

Conclusions.

1) The determination of formaldehyde produced during a Malaprade reaction by measuring the colour produced with chromotropic acid can be carried out with an accuracy of $\pm1\%$ - 2% relative, provided the oxidation is carried out in bicarbonate medium. If the oxidation is carried out in a solution at reagent pH results are very variable and appear to be, on average, about 10% lower than those obtained in bicarbonate solution.

2) A simultaneous determination of periodate consumption and formaldehyde produced during a Malaprade reaction

showed that the former determination was very accurate
and an average relative error of $\pm$ 1-2% was obtained.
The determination of formaldehyde however, although
generally within $\pm$5%, occasionally fell to $\pm$ 20-25%.
Although this accuracy is perfectly adequate for
diagnosis of structure it is of little value for
purely analytical purposes. The fall in accuracy of
the formaldehyde determination appears to be due to the
starch indicator solution.

3) The fact that the hypoiodite procedure, when used for
the determination of formaldehyde after the determin-
ation of the acidity and periodate consumption, gave
recoveries of between 90-95% generally, might be
due to two facts :

a) The formic acid determination involved addition
of alkali, followed by back titration of the excess
with acid. The presence of the alkali might have
caused hydrolysis of any formate ester, which
normally would cause low results, and would
explain the slightly higher recoveries of formic
acid and formaldehyde obtained by that method
than by other procedures.

b) The addition of bicarbonate to the oxidation
mixture prior to the periodate titration might
have a similar effect to (a).

Generally speaking all the results obtained on the
submicro scale have echoed results obtained by other workers
on other scales, but for the best results for both analytical
and diagnostic purposes the following procedures are
recommended:

1) The formic acid and periodate consumption can be
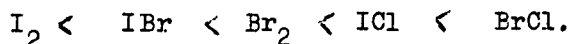determined with an accuracy of $\sim$ $\pm$2% relative using

a back-titration technique with methyl red indicator for the acid, and titration of the periodate with arsenite in buffered solution in the presence of iodide both on the same sample solution.

2) When a separate sample is used, and oxidation is done in a bicarbonate medium, the formaldehyde can be determined by measurement of its colour produced with chromotropic acid with a relative accuracy of $\pm$ 1-2%.

Such a procedure would involve 60-100 µg of sample or less, which is a reduction of ca. 10,000 times the sample normally used.

IV. The Determination of Olefinic Unsaturation.

Halogenation reagents are, from an analytical standpoint, the most convenient for the determination of olefinic/ethylenic unsaturation. The order of increasing reactivity of the various halogens in the addition to the double bond is as follows:

$$I_2 < \quad IBr \quad < \quad Br_2 \quad < \quad ICl \quad < \quad BrCl.$$

The analytical convenience of the halogens is to some extent offset by three main disadvantages. Firstly the reagents are volatile, secondly substitutive side-reactions may occur, and thirdly, less reactive olefines do not undergo addition of halogens to the double bond.

However, because other possible methods appeared to offer even greater disadvantages, it was decided to attempt to develop a reasonably general method for the determination of olefinic unsaturation using a halogenation reagent, and to couple this with an ancillary method which would take care of the less reactive olefines.
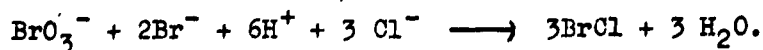
It was decided to compare two halogenation reagents :-

1) Tribromide ion in methanol. This reagent originally studied by Kaufmann (Kaufmann,H.P., Z.Untersuch.Lebensm.,

1926, 51, 3) has the advantage that the volatility of bromine is decreased whilst the tribromide ion is only slightly less reactive than bromine itself.

2) Bromine monochloride. Schulek (Schulek,E., and Burger, K., Z. Analyt.Chem.) has applied this reagent to the analysis of a variety of unsaturated compounds with satisfactory results.

1) <u>Tribromide ion</u>:- The results obtained from the analysis of several α,β-unsaturated acids and esters using this reagent were unsatisfactory. The problem is that the -COOH, or -COOEt group deactivates the double bond to such an extent that the addition of bromine proceeds extremely slowly. In order to overcome this effect it is necessary to correct the -COOH or -COOEt to the carboxylate anion. The increase in volume when the neutralisation or hydrolysis was carried out on the submicro scale resulted in the bromine concentration being far too low. It was impossible to avoid this by increasing the bromine concentration, and it was essential to obtain a significant difference between sample and blank titrations. The study of this reagent was abandoned.

2) <u>Bromine monochloride</u>:- Bromine monochloride is a far more reactive reagent than bromine itself, and will add to the double bond in α,β-unsaturated acids, without the need for prior neutralisation. The disadvantage of this reagent is inherent in its extreme reactivity, i.e. the more reactive olefines tend to 'over-brominate' unless the excess of bromine monochloride is carefully controlled.

Acidification of a bromate/bromide mixture with hydrochloric acid proceeds as follows.

$$BrO_3^- + 2Br^- + 6H^+ + 3Cl^- \longrightarrow 3BrCl + 3H_2O.$$

Accordingly three types of procedure were examined.

i) Addition of a bromate/bromide solution to the olefinic compound, and then acidification with hydrochloric acid, i.e. BrCl liberated *in situ*.

ii) A similar procedure except that the reaction vessel was evacuated before addition of the hydrochloric acid through the funnel-shaped neck of the reaction flask by releasing the stopper slightly. The use of a slight vacuum lowered the volatility of the bromine chloride.

iii) Addition of a standard bromine chloride solution to the olefinic compound (at atmospheric pressure).

The most promising results were initially obtained with procedure (i) (procedure ii gave more reproducible blanks, but the slightly improved accuracy was not offset by the increased technical difficulty). However, when it was discovered that by carrying out the determination at a temperature of 0°C the losses of bromine monochloride from a standard bromine monochloride solution were greatly reduced, it was decided to adopt this method as a general practice.

General Method

i) <u>Known Samples</u>.

The sample is placed in the submicro flask (standard type) and dissolved in measured volume of methanol, (0.10 ml.). After cooling in ice an amount of 0.01N standard bromine chloride solution is added, and the sample allowed to react for between 5 minutes and 1 hour (depending on the nature of the sample). 0.5 ml of distilled water is then added, together with 1 drop of 15% potassium iodide solution, and the liberated iodine titrated with 0.01N standard thiosulphate solution. For some samples a mercuric sulphate catalyst is required (prepared from precipitated HgO), but this must be ascertained by experiment as some compounds give very high results when Hg(II) is present.

ii) Unknown Samples.

The first step in the analysis of an unknown olefinic compound consists of preparing a series of samples containing varying mole ratios of BrCl:sample, and after having allowed sufficient time for the addition to occur (1 hour is usually ample) the excess BrCl is determined by iodometry.

This will give an indication of the olefinic content of the molecule together with the nature and extent of any side-reactions. In some cases the consumption of BrCl is constant at mole ratios $> 1.5:1$ so that if a graph is plotted of bromine chloride consumed against mole ratio of BrCl:sample the curve becomes horizontal, whilst in others the over-bromination is so marked that the curve continues to rise only slightly less steeply than in the initial portion with a slight plateau around the 2.0 or 1.5:1 values.

This exploratory examination then enables a more accurate determination to be carried out. A further series of samples containing mole ratios of bromine chloride to sample corresponding to a point on the previous graph where the curve became horizontal (i.e. mole ratios between 1.25 and 1.75:1) are prepared and allowed to react for varying lengths of time. The graph of BrCl consumption as a function of reaction time is plotted and extrapolation of the two straight portions of the curve will give an accurate value for the olefinic content.

Results.

The following compounds have been analysed successfully. Maleic, fumaric, cinnamic, crotonic, itaconic, elaidic and sorbic acids; allyl thiourea; 3:6 methylene tetrahydrophthallic acid; 3:6 methylene tetrahydrophthalimide; citral; cinnamaldehyde; trans stilbene.

The results shown in the following are typical (calculated on basis of percentage of $\overset{|}{C} = \overset{|}{C}$ group in molecule).

Table of Results

(All results are given in order of increasing reaction time)

1. Maleic acid. (Sample range 40 ——→80 µg).
   Reaction time ½ ——→ 1 hr. (Hg^{II} catalyst)
   % $\diagup C = C \diagdown$ (relative). 101.3, 104.0, 97.7, 99.1,
   97.5, 97.0.

2. Cinnamic acid. (Sample range 40 ——→ 100µg)
   Reaction time 15 min. ——→ 1 hr.
   % $\diagup C = C \diagdown$ (relative). 97.3, 99.9, 97.5, 98.9, 98.3, 96.7.

3. 3:6 Methylene tetrahydrophthallic acid. (Sample range
   40 ——→ 100 µg) (Hg^{II} catalyst - but very little effect).
   % $\diagup C = C \diagdown$ (relative). 97.9, 100.4, 87.8, 100.7, 98.6,
   93.2

4. Cinnamaldehyde. Samples from standard solution ≡ 66.08µg.
   Reaction times. 15 min. ——→ ½ hr (-9 ——→ -5 °C)
   % $\diagup C = C \diagdown$ (relative). 10.17, 102.9, 97.1, 100.9,
   106.0, 106.9.

Conclusions. The method as presented is capable of analysing a wide range of olefinic compounds, and with suitable modification can be even further extended. Very reactive olefines can be titrated directly, whilst α,β-unsaturated esters can be determined after hydrolysis.

Olefines which contain a strongly electron-attracting group, such as -C≡N, -COEt, conjugated to the double bond, cannot be determined with bromine chloride. Work is at present in progress to develop a method based on the use of morpholine, (Critchfield, F.E. Johnson, J.R., and Funk, G.L., Anal.Chem., 1956, 28, 76) a secondary amine, which adds to the double bond. Initial results have been promising, but further work is required to find an indicator more suitable for the submicro procedure.

Personnel utilized	Man-hrs.
B. Fleet and G. Dryhurst.	4,200
Mrs. M. Clarke, clerical and typing	360
Mr. R.W. Dackus, glassblower	1100

Expenditure over one year	£ " s " d		
Salaries	1,000.0		0
Glassblower	40	0	0
Typist	75	0	0
Expendable materials	94	6	6
Overheads	175	0	0
	£1,384	6	6

Expenditure : £1,384. 6. 6d.

Deficit from
last year   :   26. 0. 0d.

£1,410. 6. 6d.

Permitted expenditure ... £1,410. 0.0d.
Actual expenditure ...... £1,410. 6.6d.

Deficit :   6.6d.